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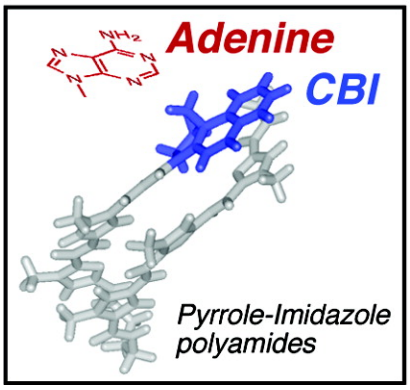

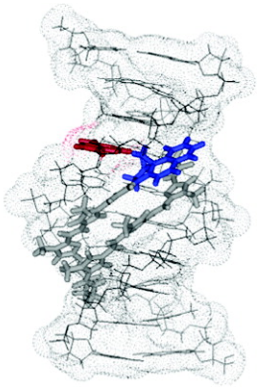

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Specific Adenine Alkylation by Pyrrole−Imidazole CBI Conjugates

Toshikazu Bando, Akihiko Narita, Shunta Sasaki, and Hiroshi Sugiyama*

Contribution from the Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo, Kyoto, 606-8502, Japan

Received April 14, 2005; E-mail: hs@kuchem.kyoto-u.ac.jp

Abstract: We examined DNA alkylation by pyrrole (Py)−imidazole (Im) hairpin polyamides, which possess 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) or cyclopropapyrroloindole (CPI) as DNA alkylating moieties. High-resolution denaturing gel electrophoresis revealed that alkylation by CBI conjugates **2** and **4** occurred specifically at adenines (A) in matched sequences, whereas CPI conjugates **1** and **3** alkylated both A and guanines (G) in matched sequences. The origin of the different reacitivity of CBI and CPI conjugates is discussed in relation to the electrophilicity of the cyclopropane moiety. The high selectivity of the CBI conjugate gives additional sequence specificity relative to CPI conjugates that would be useful for the biological applications.

Introduction

Many diseases, including malignant lymphomas and cancers, are now better understood at a genomic level. For instance, new types of anticancer agents, such as STI-571 (Glivec), that target mutated gene products show great promise in medicine. This drug targets the Abelson leukemia viral oncogene (ABL) kinase in patients with chronic myelogenous leukemia.[1] Such a design of drugs is an attractive goal in biomedicine. DNA alkylating agents, which include well-known anticancer agents such as nitrosoureas and alkyl sulfonates, are routinely used in antitumor treatments. However, these DNA damaging drugs are extremely toxic. One important question is whether the introduction of sequence selectivity to an alkylating agent can enhance its efficiency in acting against cancer cells and help to reduce the side effects on normal cells. To address this question, we have synthesized various types of conjugates between the alkylating moiety of duocarmycin A[2] or DU-86[3] and *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) polyamides.[4]

We have demonstrated that the insertion of a vinyl linker between the alkylating moiety and the Py−Im polyamides dramatically enhances both DNA alkylating activity[5] and cytotoxicity against human cancer cell lines.[6] We found that the differences in sequence specificity among these polyamides give rise to significantly different cytotoxicities in 39 human cancer cell lines.[7] Using two experimental systems, we demonstrated that alkylation of the Py−Im polyamides, which recognize specific sites on the template strand in the coding region, effectively produces a truncated mRNA in an in vitro transcription system[8] and induces sequence-specific gene silencing in human cell lines.[9] With the aim of developing antitumor agents, we introduced the *S*-enantiomer of 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI)[10] to an alkylating agent and found that it leads to higher DNA alkylation and cytotoxicity than those of the corresponding segment A of the DU-86 conjugate.[11] Further examination of CBI conjugates demonstrated that this CBI conjugate selectively alkylates adenines (A) in matched sequences, whereas the corresponding cyclopropapyrroloindole (CPI) conjugate alkylates adenines (A) and guanines (G) in matched sequences.

Results and Discussion

Synthesis of Conjugates 1−4. Compounds **1**−**4** shown in Figure 1 were synthesized by our general method of alkylating hairpin polyamides. Compounds **1** and **2** have ImImPy-γ-ImPy, and compounds **3** and **4** have ImImPyPy-γ-ImPyPy, as DNA binding moieties, respectively. Compounds **1** and **3** have a segment A of DU-86 (CPI), and **2** and **4** have CBI as their alkylation moiety. After purification using HPLC, four types of the hairpin polyamide CPI and CBI conjugates **1**−**4** were employed in the DNA alkylation experiments.

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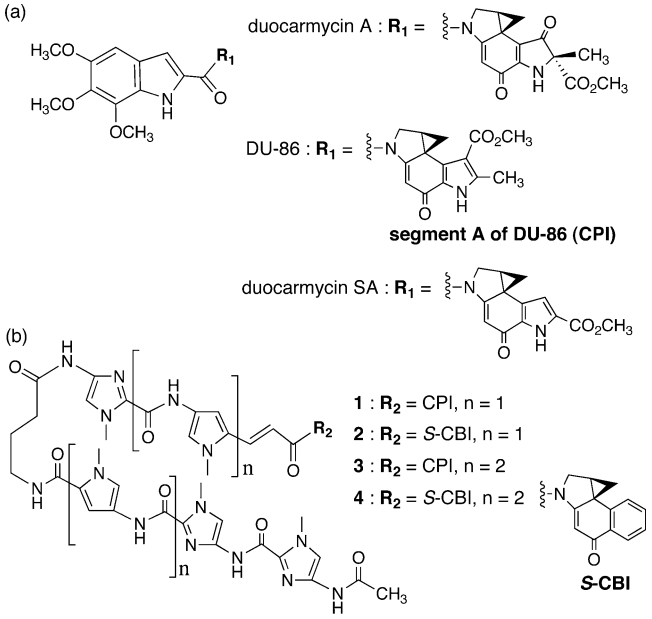

Figure 1. (a) Structures of duocarmycin A, DU-86, and duocarmycin SA. (b) Structures of *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) CPI or CBI conjugates **1**−**4**.

Evaluation of the DNA Alkylation by 1−4. Sequence-specific DNA alkylation by compounds **1** and **2** was examined on 5′-Texas Red-labeled DNA fragments, 993 bp from pQBI63 and 1065 bp from pGL-3. DNA alkylation by compounds **3** and **4** was examined on 5′-Texas Red-labeled DNA fragments, 450 bp from pUC18. Specificity was analyzed using an automated DNA sequencer. Alkylation by compounds **1**−**4** was carried out at 23 °C for 6 h, followed by quenching with an excess of calf thymus DNA. The samples were heated at 94 °C under neutral conditions for 20 min. Sequences of the alkylated regions were determined by thermal cleavage of the DNA strand at the alkylated sites. Under these heating conditions, all A and G residues containing N3-alkyl adducts are cleaved quantitatively to produce an electrophoretic band upon heating under these conditions.[5,11,12] Sequences of the alkylated 1065 bp DNA fragment and 993 bp DNA fragment by compounds **1** and **2**, after heat treatment, are shown in Figure 2a,b.

In both DNA fragments, alkylation by the CPI conjugate **1** occurred specifically at the sequences 5′-WGCC**A/G**-3′ (where W = A or T), with a faint 2-bp mismatch alkylation at sites 4 and 5. No significant preferences for A and G alkylation sites were found. Interestingly, DNA alkylation by the corresponding CBI conjugate **2** occurred exclusively at sequences 5′-WGCC**A**-3′. The absence of alkylation at site 4 by conjugate **2** indicates that the CBI conjugate reduces mismatch alkylations. Densitometric analysis after denaturing gel electrophoresis demonstrated that alkylation at 5′-WGCC**G**-3′ was not detected at sites 3, 9, 10, or 14 (Figure 2c−g). The correlation between alkylation efficiency and concentration of **1** was biased due to extensive DNA alkylation. In particular, more than half of the starting DNA was consumed in the experiment shown in lanes 2, 3, 9, and 10.

To explore the generality of A/G selectivity of the CPI and CBI conjugates, we examined DNA alkylation by the longer Py−Im conjugates, **3** and **4**. Figure 3 shows the sequencing gel analysis of a 450 bp DNA fragment alkylated by compounds **3** and **4** after heat treatment. Alkylation by the CPI conjugate **3** occurred at the A and G sites of the match sequences of 5′-WGWCC**A/G**-3′, together with a minor mismatch alkylation at the G site of 5′-T*T*ACC**G**-3′ (lanes 2−5). By contrast, alkylation by CBI conjugate **4** occurred only at the A of the matching sequence, 5′-AGTCC**A**-3′. Because DNA alkylation was assumed to be complete under these conditions, the level of A/G selectivity by the CBI conjugates is at least 10-fold higher, judging from the densitometric analysis of the gel electrophoresis.

HPLC Analysis of DNA Alkylation by Conjugates 1 and 2. To further confirm the A/G alkylating selectivity by the CPI and CBI conjugates, we investigated the alkylation of the duplex decamer 5′-CAAGCCAGAG-3′ (ODN1)/5′-CTCTGGCTTG-3′ (ODN2) and 5′-GTTGCCGTCA-3′ (ODN3)/5′-TGACGGCAAC-3′ (ODN4), which were designed according to the results of the gel electrophoresis experiments described previously. In accord with a previous observation,[5] the results showed that both duplexes of ODN1/ODN2 and ODN3/ODN4 were alkylated by CPI conjugate **1** at levels of 81 and 65%, respectively, after a 5 min incubation (Figure 4a). Similarly, ODN1/ODN2 was alkylated by CBI conjugate **2** to 53%, while conjugate **2** provided a very small peak of the adduct (less than 1%) using ODN3/ODN4 (Figure 4b). These results confirmed a significantly lower reactivity of compound **2** for G than that of compound **1**. The level of A/G selectivity by CBI in this system was estimated as higher than 50-fold.

Presumed Origin of Specific Adenine Alkylation by CBI Conjugate. It is generally accepted that the nucleophilicity of the N3 of A is higher than that of the N3 of G, and therefore, minor groove-binding antibiotics such as CC-1065,[13] duocarmycin A, and DU-86 alkylate the N3 of A at the 3′ ends of consecutive AT sequences. CPI and CBI analogues of duocarmycin A also predominantly alkylate the N3 of A.[14] These results indicated that alkylation at N3 of G by minor groove alkylating agents is very rare. However, duocarmycin A alkylates N3 of G when the oligonucleotide substrate does not have an A at the 3′ ends of consecutive AT sequences, indicating that duocarmycin A has the potential to react with the N3 of G.[15] Indeed, we found that duocarmycin A alkylates the N3 of G when it forms a heterodimer with distamycin A[16] or pyrrole (Py)−imidazole (Im) triamides.[17] Asai et al. investigated the reaction of calf thymus DNA with duocarmycin analogues. They found that the ratio of A and G alkylation correlates with the electrophilicity of the compounds and was with duocarmycin A, 2:1, DU-86, 10:1, and duocarmycin SA, only the A adduct, when high concentrations of the drug were used (drug/DNA bp = 1/20).[18] These results clearly indicate that lowering the

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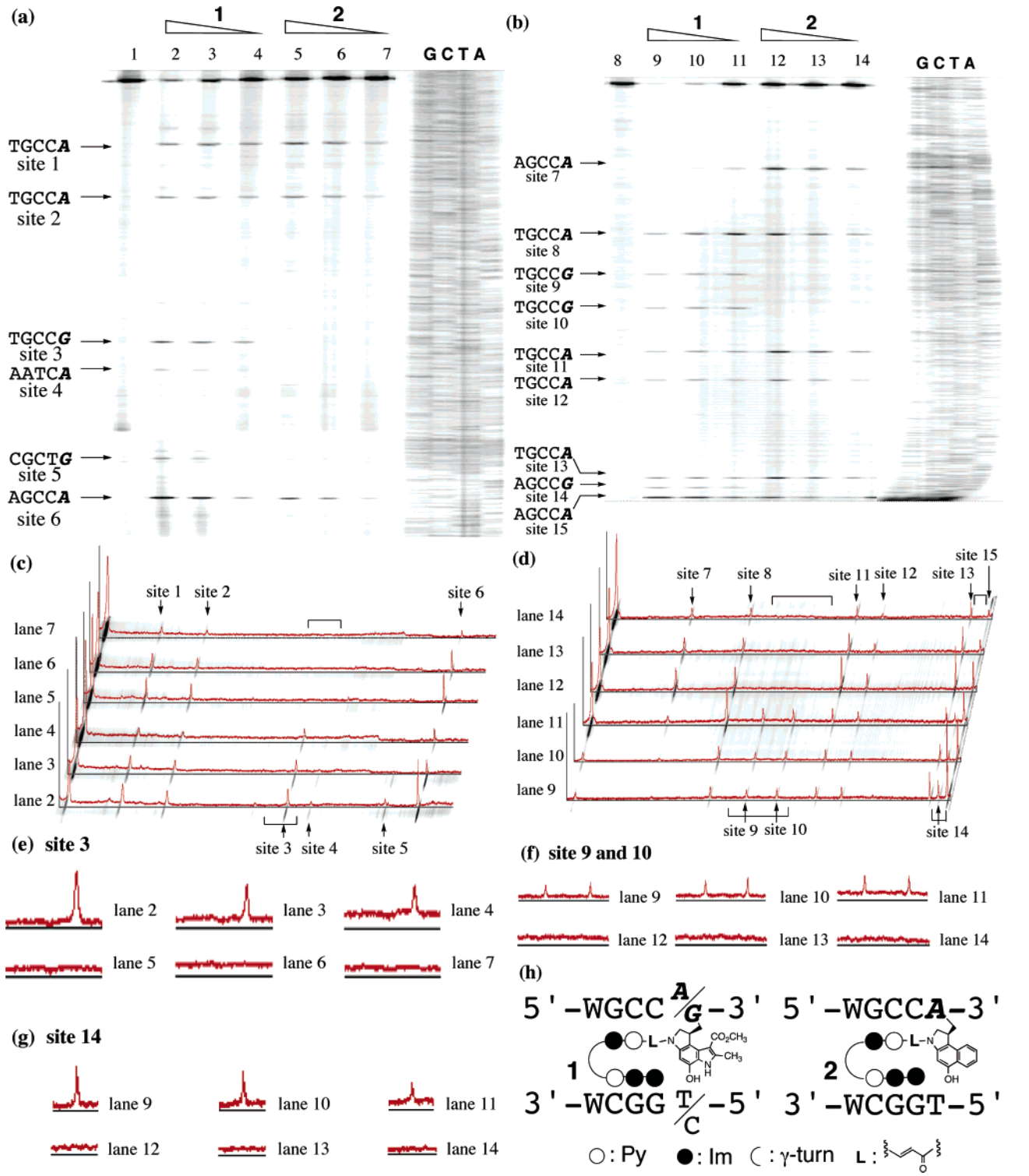

Figure 2. Comparison of thermally induced strand cleavage of 5′-Texas Red-labeled DNA fragments alkylated by compounds **1** and **2**. (a) Alkylation of a 1065 bp DNA fragment by compounds **1** and **2**. Lane 1: DNA control; lanes 2−4: 50, 25, 12.5 nM compound **1**; and lanes 5−7: 50, 25, 12.5 nM compound **2**. (b) Alkylation of a 993 bp DNA fragment by compounds **1** and **2**. Lane 8: DNA control; lanes 9−11: 25, 12.5, 5 nM compound **1**; and lanes 12−14: 50, 25, 12.5 nM compound **2**. The arrows indicate the sites of alkylation. Densitometric analyses of lanes 2−7 (c) and lanes 9−14 (d) are shown in red. (e) Expansion of site 3 in lanes 2−7. (f) Expansion of sites 9 and 10 in lanes 9−14. (g) Expansion of site 14 in lanes 9−14. (h) Schematic representation of alkylation by compounds **1** and **2** at the target sequences, 5′-WGCCPu-3′ and 5′-WGCCA-3′.

electrophilicity of the cyclopropane moiety of duocarmycin analogues increases the A selectivity. Importantly, we observed that Py−Im polyamide conjugates possessing segment A of duocarmycin A, or DU-86 as an alkylating moiety, alkylate both A and G of matched sequences with similar efficiency.[5,12,19] Thus, strong binding of the Py−Im polyamide conjugate with a vinyl linker possessing optimal orientation of the cyclopropane ring facilitates the reaction of CPI with the N3 of both A and

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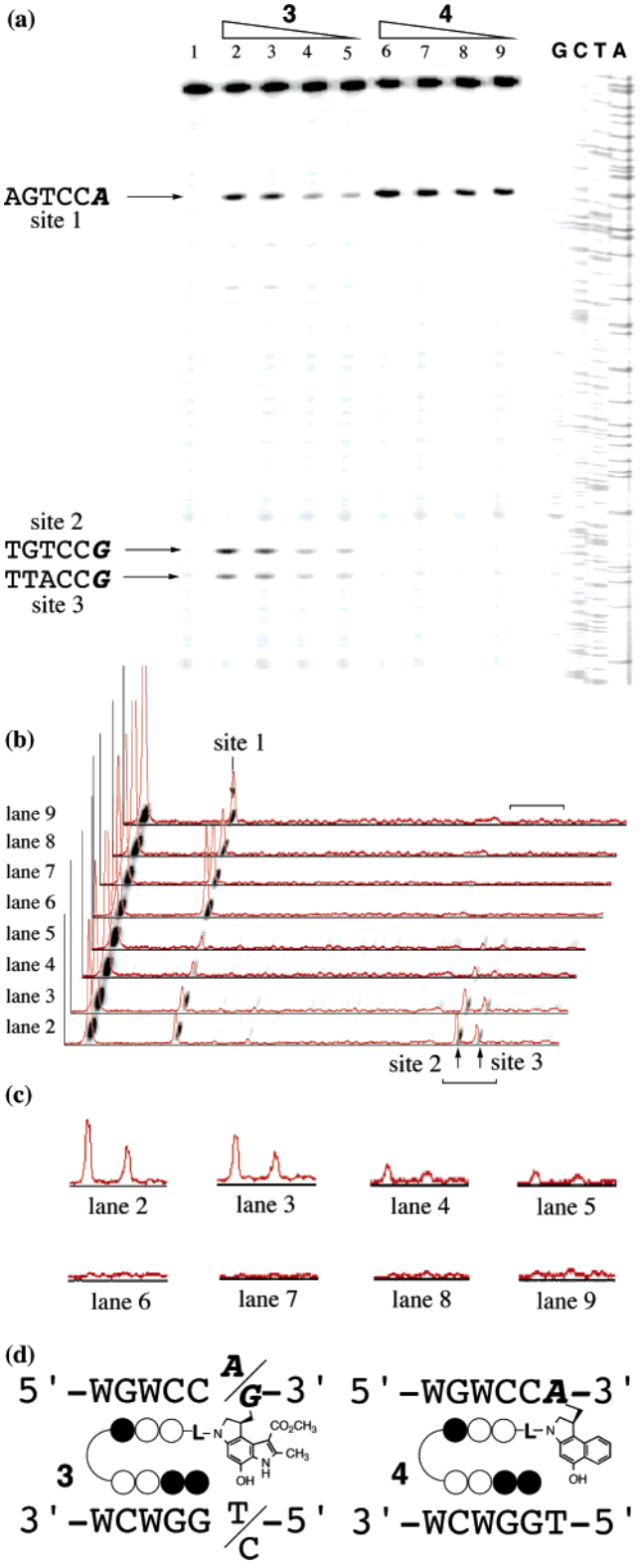

Figure 3. Comparison of thermally induced strand cleavage of 5′-Texas Red-labeled DNA fragments alkylated by compounds **3** and **4**. (a) Alkylation of 450 bp DNA fragment by compounds **3** and **4**. Lane 1: DNA control; lanes 2−5: 50, 25, 12.5, 6.25 nM compound **3**; and lanes 6−9: 50, 25, 12.5, 6.25 nM of compound **4**. The arrows indicate the sites of alkylation. (b) Densitometric analyses of lanes 2−9 are shown in red. (c) Expansion of sites 2 and 3 in lanes 2−9. (d) Schematic representation of alkylation by compounds **3** and **4** at their target sequences, 5′-WGWCCPu-3′ and 5′-WGWCCA-3′.

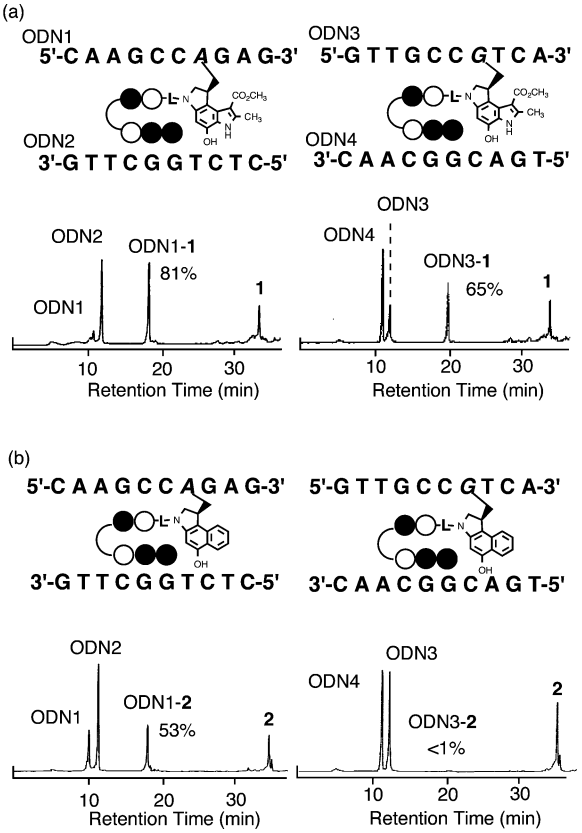

Figure 4. HPLC analysis and schematic representation of alkylation of ODN1/2 and ODN3/4 by compounds **1** (a) and **2** (b).

G. Although other types of hairpin CBI conjugates with flexible linkers alkylated several As of both strands,[20] we expected that the CBI conjugates with a vinyl linker would react with both A and G. Surprisingly, Py−Im CBI conjugates (**2** and **4**) specifically undergo alkylation at N3 of A despite having a similar binding orientation to the corresponding CPI conjugate, as discussed later.

To investigate the electrophilicity of the cyclopropane moiety of CPI and CBI conjugates, we evaluated the stability of the model compounds ImPyCPI, **5** and ImPyCBI, **6** at pH 7.0 and 37 °C. HPLC analysis of the incubated solutions indicated the formation of a hydrolyzed product **7** or **8** as a major detectable product via nucleophilic addition of water at the C4 or C9 position with significantly different rates (Figure 5 and Scheme 1).

HPLC analyses demonstrated that the half-lives of **5** and **6** under the conditions stated are 53 and 330 h, respectively. These data are consistent with those reported previously for *N*-Boc protected CPI and CBI derivatives.[21] The data suggest that lower electrophilicity of the CBI conjugate could be responsible for the selective alkylation at A in the DNA minor groove.

Binding Model of 1 and 2 to Target Oligonucleotides. To gain an insight into the location of the hairpin polyamides **1** and **2** in the DNA minor groove, we constructed ODN3/ODN4−hairpin polyamide complexes based on the ^{1}H NMR structure of the DNA binding complexes.[22] The energy-minimized structures of the ODN3/ODN4-**1** and -**2** complexes suggest that

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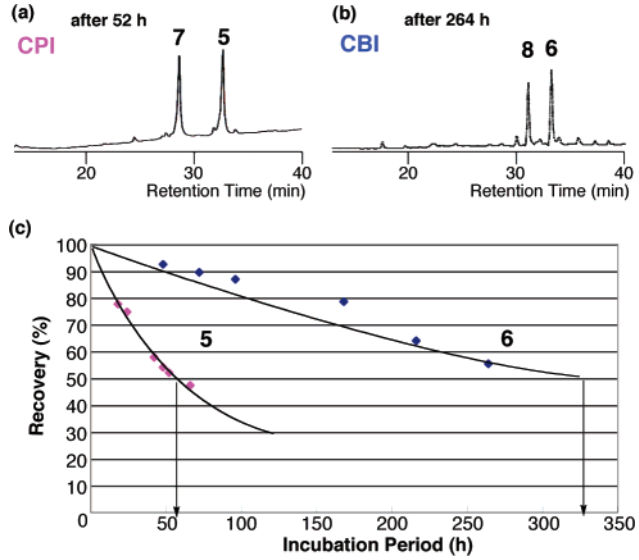
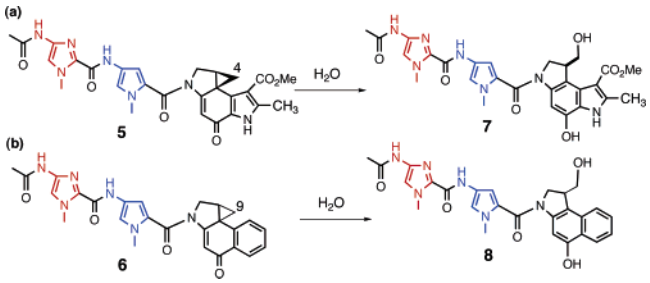


Figure 5. HPLC analysis of hydrolysis of (a) ImPyCPI, **5** and (b) ImPyCBI, **6**. (c) Time course of hydrolysis of **5** and **6**. Recovery (%) of **5** (purple diamond) and **6** (blue diamond).

Scheme 1



binding of **1** and **2** to the minor groove of ODN3/ODN4 is substantially the same in that the cyclopropane subunit of CPI or CBI is located in close proximity to the nucleophilic N3 of the G_7 residues: ODN3/ODN4-**1**, 3.42 Å with the angle of the N3−C4−C3b bond = 143.16°, and ODN3/ODN4-**2**, 3.29 Å with the angle of the N3−C9−C8b bond = 151.35° (Figure 6). The similar binding orientations of the CPI and CBI moieties and the lack of obvious steric hindrance between the 2-amino group of G_7 and CBI suggest that the different reactivity would be imparted by the electrophilicity of cyclopropane.

Conclusions

We found that DNA alkylation by the CPI conjugates (**1** and **3**) occurs both at A and G, whereas Py−Im CBI conjugates (**2** and **4**) specifically undergo alkylation at A despite having a similar binding orientation to the corresponding CPI conjugate. This additional sequence selectivity given by the CBI-conjugated hairpin alkylating polyamides provided extensions of the target sequence and reductions of mismatch alkylations that substantially improves selectivity to the target sequence. Boger's group has investigated the stability and cytotoxicity of various duocarmycin derivatives and found that the order of stability is CPI < CBI and that the compounds with higher stability at neutral pH have higher cytotoxicity.[23] In fact, the cytotoxicity

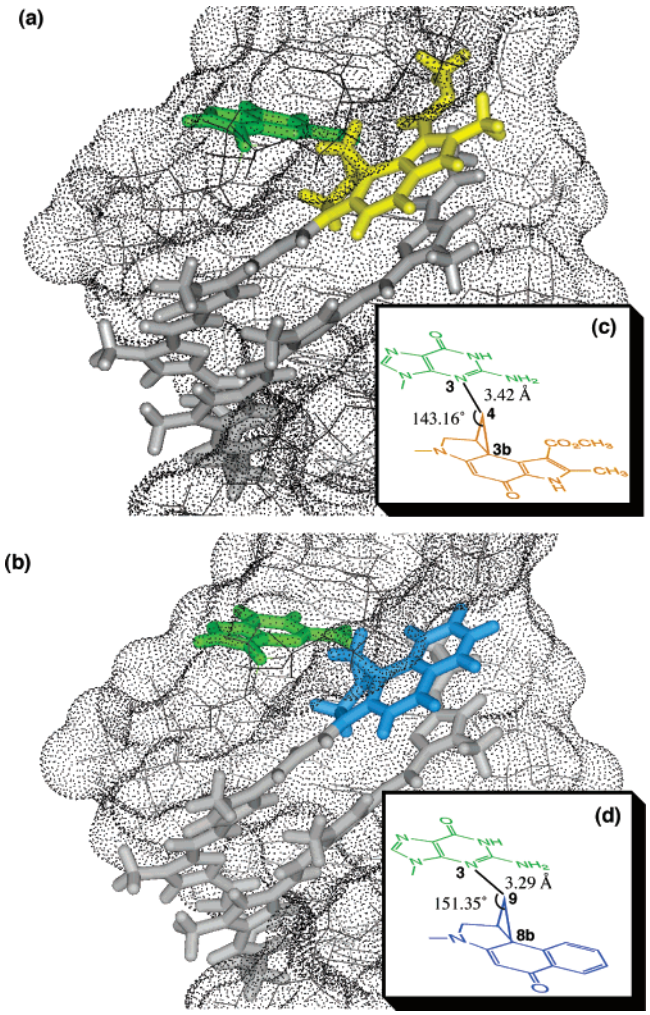


Figure 6. Energy-minimized structure of d(GTTGCCGTCA) ODN3/d(TGACGGCAAC) ODN4-**1** (a) and -**2** (b). Schematic representation of distance and angle of N3 of guanine and CPI (c) and CBI (d).

of CBI conjugate **4** against 39 human cancer cell lines was 1 order of magnitude higher than that of the deacetyl amino derivative of **3**.[11] These results suggest the CBI conjugates possessing A alkylation specificity would be useful for biological applications. These advantages of the CBI motif will make the DNA-alkylating Py−Im polyamides more potent and will thus help the development of tailor-made anticancer drugs, which could thereby be used to target unique sequences in the human genome.

Experimental Procedures

General Methods. Reagents and solvents were purchased from standard suppliers and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel 60 plates impregnated with a 254 nm fluorescent indicator (from Merck). Plates were visualized by UV illumination. ^{1}H NMR spectra were recorded with a JEOL JNM-A 500 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was produced on a BioTOF II (Bruker Daltonics) mass

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spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA sequencer, and data were analyzed by FRAGLYS version 2 software (HITACHI Inc.). Ex Taq DNA polymerase and Suprec-02 purification cartridges were purchased from Takara Co.; the Thermo Sequenase core sequencing kit and loading dye (dimethylformamide with fuschin red) were from Amersham Co. Ltd; 5′-Texas-Red-modified DNA oligonucleotides were from Kurabo Co. Ltd; and 50% Long Ranger gel solution was from FMC Bioproducts. P1 nuclease and calf intestine alkaline phosphatase (AP, 1000 units/mL) were purchased from Roche Diagnostics. *S*-1,2,9,9a-Tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (*S*-CBI) was synthesized and separated by reported methods.[10] The purities of 5-(benzyloxy)-3-(*t*-butyloxycarbonyl)-1-(hydroxymethyl)-1,2-dihydro-3H-benz[*e*]indole and (*R*)-*O*-acethyl mandelate ester (1*S*,2′*R*) were more than 99%, as determined by HPLC. Conjugates **1**−**4** were prepared by the reported procedures.[5,11] Spectral data for compounds **3**−**5** were previously reported.[6,11]

AcImImPy-γ-ImPyLCPI (1). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.83 (brs, 1H), 10.29 (s, 2H), 10.26 (s, 2H), 9.91 (s, 1H), 9.86 (s, 1H), 8.03 (brt, 1H), 7.55 (s, 1H), 7.49 (s, 1H), 7.46 (d, J = 15.0 Hz, 1H), 7.38 (s, 1H), 7.34 (s, 1H), 7.21 (s, 1H), 6.98 (s, 1H), 6.89 (s, 1H), 6.86 (s, 1H; CH), 6.60 (d, J = 15.0 Hz), 4.15 (m, 1H), 4.08 (m, 1H), 3.99 (s, 6H), 3.97 (s, 3H), 3.89 (s, 3H), 3.80 (s, 3H), 3.70 (s, 3H), 3.20 (m, 2H), 2.45 (s, 3H), 2.35 (m, 2H), 2.32 (m, 1H), 2.03 (s, 3H), 1.80 (m, 2H), 1.22 (m, 1H); ESI-TOF MS *m*/*z* calcd for $C_{49}H_{53}N_{16}O_{10}$ $[M + H]^+$ 1025.4131; found 1025.4157.

AcImImPy-γ-ImPyLCBI (2). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.28 (s, 1H), 10.25 (s, 2H), 9.98 (s, 1H), 9.32 (s, 1H), 8.04 (brt, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.60 (d, J = 15.0 Hz, 1H), 7.55 (s, 2H), 7.49 (s, 1H), 7.45 (s, 1H), 7.42 (m, 1H), 7.41 (s, 1H), 7.20 (s, 1H), 7.18 (m, 1H), 7.00 (d, J = 8.0 Hz, 1H), 6.99 (s, 1H), 6.58 (d, J = 15.0 Hz, 1H), 4.33 (dd, J = 10.0, 5.0 Hz, 1H), 4.27 (d, J = 5.0 Hz, 1H), 3.99 (s, 3H), 3.97 (s, 3H), 3.93 (s, 3H), 3.79 (s, 3H), 3.72 (s, 3H), 3.19 (m, 3H), 2.35 (brt, 2H), 2.03 (s, 3H), 1.79 (brq, 2H), 1.71 (dd, J = 4.0, 8.0 Hz, 1H), 1.56 (t, J = 4.0 Hz, 1H); ESI-TOF-MS *m*/*e* calcd for $C_{48}H_{50}N_{15}O_8$ $[M + H]^+$ 964.3967; found 964.4017.

ImPyCBI (6). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.65 (s, 1H), 8.14 (d, J = 7.0 Hz, 1H), 7.54 (s, 1H), 7.45 (t, J = 7.0 Hz, 1H), 7.34 (t, J = 7.0 Hz, 1H), 7.32 (s, 1H), 7.17 (d, J = 2.0 Hz, 1H), 6.84 (d, J = 7.0 Hz, 1H), 6.60 (d, J = 2.0 Hz, 1H), 6.40 (s, 1H), 4.22 (dd, J = 5.0 Hz, 11.0 Hz, 1H), 4.02 (d, J = 11.0 Hz, 1H), 3.98 (s, 3H), 3.79 (s, 3H), 2.72 (m, 1H), 2.26 (m, 1H), 2.09 (s, 3H), 1.71 (m,1H); ESMS *m*/*z* calcd for $C_{26}H_{25}N_6O_4$ $[M + H]^+$ 485.2, found 485.2.

Preparation of 5′-Texas Red-Labeled DNA Fragments. A 5′-Texas Red-labeled 993 bp DNA fragment was prepared by the previously described method. A 5′-Texas Red-modified 993-bp DNA fragment was prepared by PCR using the pQBI63 plasmid and 5′-Texas Red modified GGTGATGTCGGCGATATAGG-3′ and 20-mer primer, 5′-CCCCAAGGGGTTATGCTAGT-3′. A 5′-Texas Red-labeled 1065 bp DNA fragment was similarly prepared from pGL3 plasmid using the following primers: 5′-Texas Red-modified CTCCTCAGAAA-CAGCTCTTCTTC-3′ and 5′-GCGATCTGCATCTCAATTAGTC-3′. A 5′-Texas Red-labeled 450 bp DNA fragment was similarly prepared from pUC18 plasmid using the following primers: 5′-Texas Red-modified AGAATCAGGGGATAACGCAG-3′ and an unlabeled primer and 5′-TTACCAGTGGCTGCTGCCAG-3.′ After the filtering purification of the PCR products, their concentrations were determined by UV absorption.

High-Resolution Gel Electrophoresis. The 5′-Texas Red-labeled DNA fragments (10 nM) were alkylated by various concentration of **1**−**4** in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C for 6 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 8 μL of loading dye (formamide with fuschin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA Sequencer.

Alkylation of Oligonucleotides by Conjugates 1 and 2 as Monitored by HPLC. A reaction mixture (10 μL) containing conjugates **1** and **2** (150 μM) and the duplex oligonucleotide (100 μM duplex concentration) in 5 mM sodium phosphate buffer (pH 7.0, DMF/water = 1:9) was incubated at 23 °C for 5 min and for 1 h. The progress of the alkylating reaction was monitored by HPLC at 254 nm using a Chemcobond 5-ODS-H column (4.6 mm × 150 mm). Elution was performed with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient (0−40 min) at a flow rate of 1.0 mL/min.

Hydrolysis of Conjugates 5 and 6 as Monitored by HPLC. A reaction mixture (40 μL) containing conjugates **5** or **6** (150 μM) in 5 mM sodium phosphate buffer (pH 7.0, DMF/water = 2:8) was incubated at 37 °C. The progress of the hydrolysis was monitored by HPLC at 254 nm using a Chemcobond 5-ODS-H column (4.6 mm × 150 mm). Elution was performed with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient (0−40 min) at a flow rate of 1.0 mL/min. Hydrolyzed ImPyCPI, **7**: ESI-MS *m*/*e* calcd for $C_{27}H_{30}N_7O_7$ $[M + H]^+$ 564.2, found 564.3 and hydrolyzed ImPyCBI, **8**: ESI-MS *m*/*e* calcd for $C_{26}H_{27}N_6O_5$ $[M + H]^+$ 503.2, found 503.2.

Molecular Modeling Studies. Minimizations were performed with the Discover (MSI, San Diego, CA) program using cvff force-field parameters. The starting structure was built on the basis of the NMR structure of the ImPyPy-γ-PyPyPy-d(CGCTAACAGGC)/d(GCCTGT-TAGCG) complex[22a] and the Duo-Dist-octamer complex.[22b] The connecting parts between them were built using standard bond lengths and angles. The CBI unit of the assembled initial structure was energy minimized using a distance-dependent dielectric constant of $\epsilon = 4r$ (r stands for the distance between atoms i and j) and with convergence criteria having an RMS gradient of less than 0.001 kcal/mol Å. Eighteen Na cations were placed at the bifurcating position of the O−P−O angle at a distance of 2.51 Å from the phosphorus atom. The resulting complex was soaked in a 10 Å layer of water. The whole system was minimized without any constraint, to the stage where the RMS was less than 0.001 kcal/mol Å.

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